ORIGIN OF THE METAZOA: A REVIEW OF MOLECULAR BIOLOGICAL STUDIES WITH SPONGES

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The phylogenetic position of Porifera is near the base of the kingdom Metazoa. During the last few years rRNA sequences, and more importantly cDNAs/genes coding for proteins, have been isolated and characterised from sponges, especially from the marine demosponge *Geodia cydonium*. Analyses of their deduced amino acid sequences allowed a molecular biological approach to solve the problem of monophyly of the Metazoa. Molecules of the extracellular matrix/basal lamina, with the integrin receptor, fibronectin and galectin as prominent examples, cell-surface receptors (tyrosine kinase receptor), elements of nerve systems (crystallin, metabotropic glutamate receptor) as well as homologs/modules of an immune system (immunoglobulin-like molecules, SRCR- and SCR-repeats, Rhesus system) unequivocally classify Porifera as true Metazoa. As living fossils sponges also show pecularities not known in other metazoan phyla provided with simple, primordial molecules allowing cell-cell and cell-matrix adhesion as well as processes of signal transduction known in a more complex manner from higher Metazoa. Tissues of sponges are rich in telomerase activity, suggesting a high plasticity in the determination of cell lineages. Based on this experimental background a first successful approach to establishing a cell culture from a sponge was possible. It is concluded that molecular biological studies using sponges as models will not only help us to understand the evolution of the Metazoa from the Protista, but also the complex, hierarchial regulatory network of cells in higher Metazoa. ❐ *Porifera, evolution, monophyly, receptors, phylogeny, molecular biology, (Eu)Metazoa.*

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The transition from unicellular to multicellular organisms has taken place in all five kingdoms of life; this process took place separately in Fungi (Ascomycota), Plantae (Chlorophyta) and in Metazoa. The origin of plants appears to be well established within the phylum Chlorophyta (Margulis & Schwartz, 1995), whereas the origin of Fungi, and especially of Metazoa, is perhaps the most enigmatic of all phylogenetic problems (Willmer, 1994).

The origin of the Metazoa remained uncertain until a few years ago. At that time two questions were paramount: 1) what were the relationships between the different metazoan phyla in general, and between the lowest metazoan phylum, the Porifera (sponges), and those of higher invertebrates in particular; and 2) what are the ancestor(s) of the Metazoa among the Protista ? Some authors favoured the idea that sponges had unicellular ancestors different from those of other Metazoa [polyphyly] (Margulis & Schwartz, 1995), while other scientists (e.g. Morris, 1993), believed that multicellular animals evolved only once [monophyly].

One powerful approach particularly helpful in answering questions on the presence or absence of corresponding structures in sister groups is to gather molecular data from the respective taxa. Here, a clear distinction must be made. Nucleotide [nt] sequence data have been gathered from genes of species in different phyla, encoding small and large ribosomal RNA. These data have been used to build phylogenetic trees to resolve deep branches. The outcome in most reports was that bootstrap statistics supporting mono- or polyphyly is low (e.g. Cavalier-Smith et al., 1996), or even not at all significant (Rodrigo et al., 1994).

In a second, separate concept, amino acid [aa] sequences deduced from nt sequences from genes and proteins that are known from metazoan systems (e.g. immune, adhesion, sensory systems), have been used to obtain reliable

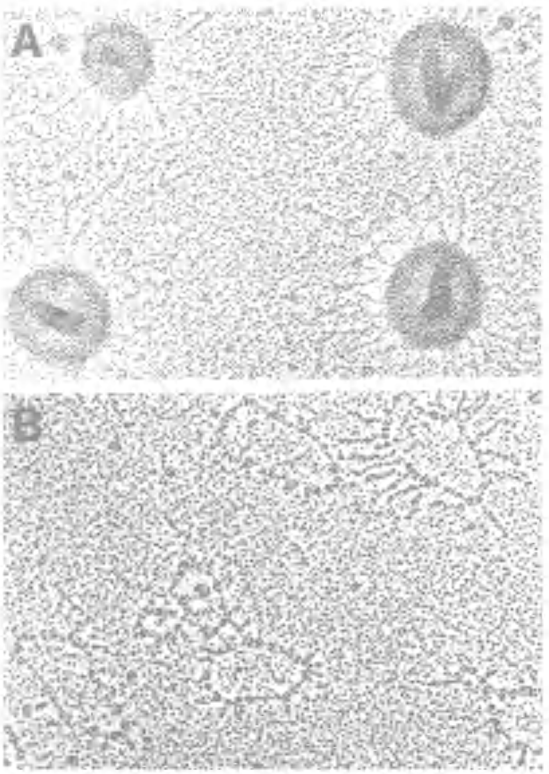

FIG. 1. Adhesion molecule of the sponge *G. cydonium*; electron micrograph. A, Native form of the aggregation factor. B, Aggregation factor core structure with the circular center and the 25 radiating arms. Preparation shadowed with platinum (magnification x140,000).

insight into the branching of metazoan phyla from a potential common ancestor (Pfeifer et al., 1993). Our research group has introduced this approach to establish the phylogenetic position of Porifera, within the Metazoa. Our data are compatible with the view that all Metazoa are monophyletic in origin (Müller et al., 1994; Müller, 1995).

We also discuss evidence for evolution of cell lineages in early Metazoa; we demonstrate the use of a new type of sponge 'organotypic' cell culture in cell proliferation and cell death; and finally we discuss evidence for the separation of the Porifera into two subphyla.

DISCUSSION

GENOME SIZE OF PORIFERA. Using the method of cytometry and DAPI staining, the DNA content of haploid cells (C-value) from *Geodia cydonium* and *Suberites domuncula* was found to be 1.7pg, corresponding to $1.7x10^6$kb (Imsiecke et al., 1995). This unexpectedly high value is further exceeded by the result which came from a separate technique, the determination of DNA reassociation kinetics. In a recent calculation, based on the determination of genetic complexity, a value of 3.3pg DNA/haploid genomic set was calculated (Bartmann-Lindholm et al., 1997). In comparison, the C value for human cells is $3.4x10^6$kb (see Li & Graur, 1991). The number of chromosomes in the diploid state in *S. domuncula* is 32 (Imsiecke et al., 1995).

The recent finding that five major subcomponents of DNA could be distinguished in *G. cydonium* by density gradient centrifugation (Bartmann-Lindholm et al., 1997) was surprising as it indicates a heterogeneity which has not been reported in any other metazoan. The genetic complexity within these subcomponents was determined by reassociation kinetics to vary from $2.1x10^8$bp to $1.4x10^9$bp, corresponding to a content of single copy DNA of >93% (Bartmann-Lindholm et al., 1997). The extreme heterogeneity in DNA composition of the genome of *G. cydonium* suggests that an unusually high exchange of well-defined DNA regions occurred in this animal.

METAZOAN GENES/PROTEINS IN PORIFERA. *Molecules/modules of the extracellular matrix/basal lamina in G. cydonium.* It is now well acknowledged that repeated sequences or modules (Patthy, 1995) are found in proteins of the complement system, extracellular matrix and also in various cell-surface receptors. Mobile modules, according to the definition of Patthy (1995), are protein-coding domains that are flanked by introns of identical phase, facilitating a dispersal within the genome. It is shown here that most polypeptides deduced from cDNA sequences from sponges are assembled by an unusually large variety of such modules.

Two types of adhesion systems have been described in sponges, cell-cell and cell-matrix systems. With respect to the first system, the aggregation factor (AF) is considered to be the major extracellular molecular complex (Fig. 1). AFs were enriched from two sponge species, *Microciona prolifera* and *G. cydonium* (reviewed in Müller, 1982). The AF is a multiprotein complex which interacts with a membrane component, the aggregation receptor (AR), that has been identified but not yet purified.

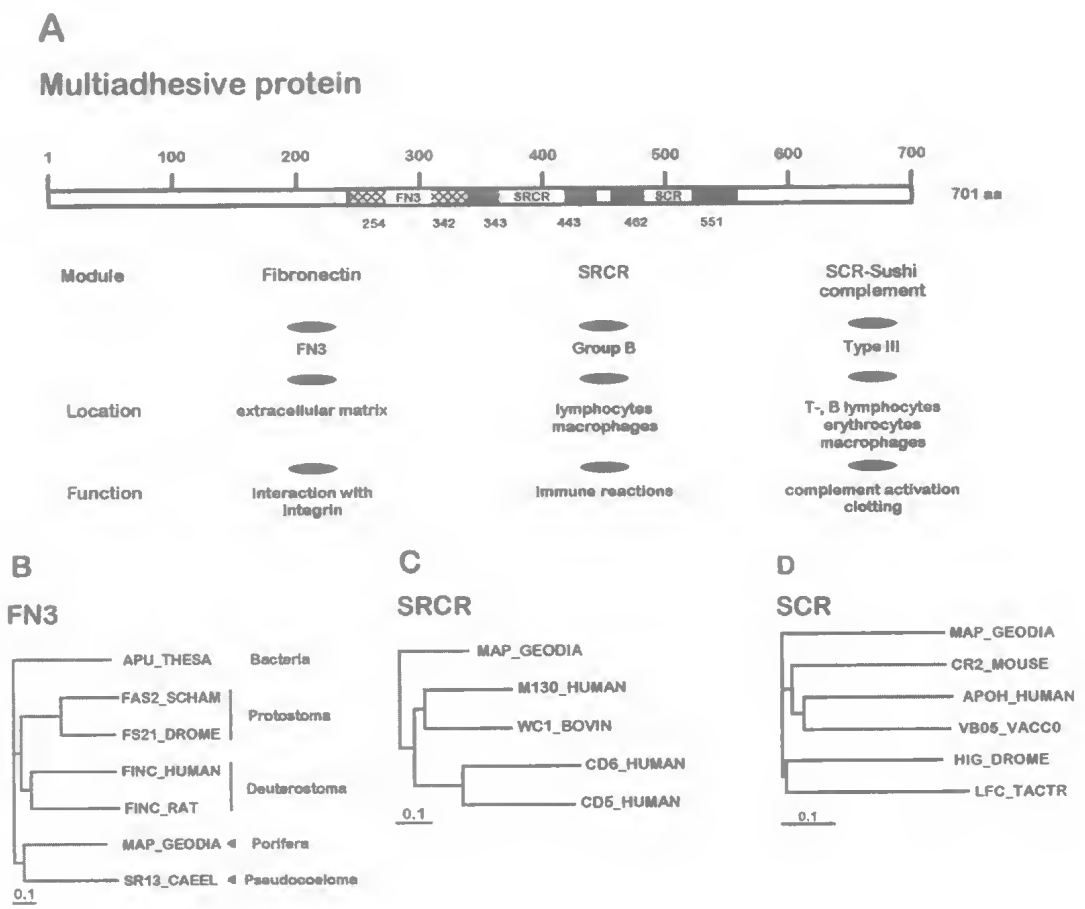

FIG. 3. A, Scheme of the putative 'multiadhesive protein' (MAP_GEODIA), cloned from *G. cydonium*. Three modules can be identified in this protein; the fibronectin- (FN3), the a scavenger receptor cysteine-rich (SRCR)- and the a short consensus repeat (SCR; Sushi) module. B-D, Phylogenetic analyses of the modules. B, Unrooted phylogenetic tree composed from the deduced aa sequences of FN3 modules found in 1) Metazoa, from deuterostomes human (FINC_HUMAN), and rat (FINC_RAT; module 6), from protostomes FN3 from *D. melanogaster* (FS21_DROME) and the arthropod *S. americana* (FAS2_SCHAM; module one), the pseudocoelomate *C. elegans* (SR13_CAEEL) and the sponge *G. cydonium* (MAP_GEODIA), as well as in 2) Bacteria *T. saccharolyticum* (APU_THESA; module one). C, Phylogenetic tree computed from five SRCR scavenger molecules of group B; the modules from the sponge MAP_GEODIA, the human CD6 antigen (CD6_HUMAN), human CD5 surface glycoprotein (CD5_HUMAN), human M130 antigen (M130_HUMAN) and bovine WC1 surface antigen (WC1_HUMAN) were analyzed. D, Phylogenetic tree constructed from SCR modules of the following six polypetides; the module from MAP_GEODIA together with the corresponding type III SCRs from human beta-2-glycoprotein I precursor (APOH_HUMAN), mouse CR2 complement receptor type 2 precursor (CR2_MOUSE), *D. melanogaster* locomotion-related protein (HIG_DROME), *Limulus* clotting factor C precursor from *Tachypleus tridentatus* (LFC_TACTR) and the host range protein precursor from the vaccinia virus strain LC16MO (VB05_VACCO). The evolutionary distance of 0.1 aa substitutions per position in the sequence is given.

matrix (ECM): 1) fibronectins, 2) collagens, and 3) galectin.

Fibronectin. Fibronectins are high molecular weight glycoproteins, present in most ECM and also in blood plasma. A typical fibronectin

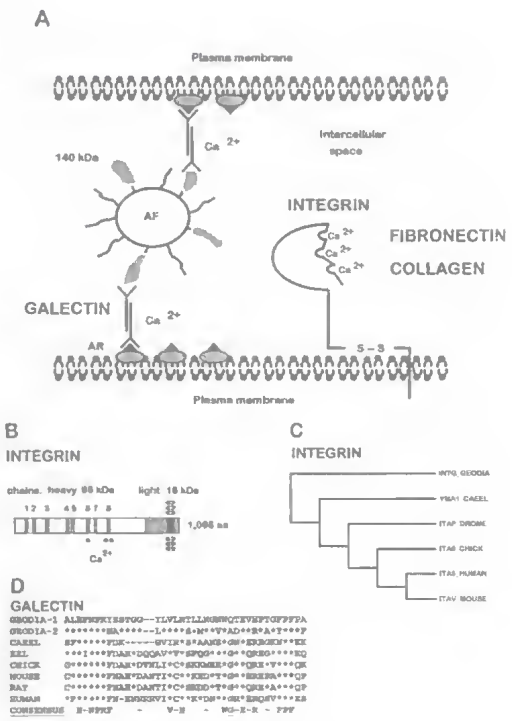

FIG. 2. A, Scheme of aggregation factor (AF)-mediated cell recognition in *G. cydonium*. A 29-kDa aggregation receptor (AR) is inserted into the plasma membrane to which one galectin molecule binds. In the presence of Ca^{2+} a second galectin molecule binds to the first one. Then these two molecules form a bridge between the AR and the 140 kDa polypeptide, associated with the AF. Following this scheme, the interactions between the AF and the AR involves the galectin which might bind to carbohydrate both at the 140 kDa polypeptide and at the AR. In addition, the sponge contains an integrin receptor which is assumed to interact with fibronectin and collagen. B, Sponge integrin receptor. Schematic presentation of the structural features in *G. cydonium* integrin α subunit. The heavy and light chains are indicated (the light chain is shaded). Positions of the 8 characteristic repeats of integrins are marked 1 to 8. Three putative Ca^{2+}-binding sites as well as the C-terminal transmembrane region are indicated. C, Dendrogram computed from the deduced aa sequences of integrin α subunits found to be most homologous to sponge integrin α (INTG_GEODIA) with the corresponding sequences from the invertebrate species, *Caenorhabditis elegans* (YMA1_CAEEL) and *Drosophila melanogaster* (ITAP_DROME), and the vertebrate species, mouse integrin αV (ITAV_MOUSE), chicken integrin αVIII (ITA8_CHICK) and human fibronectin receptor α subunit (ITA5_HUMAN). D, Multiple alignment of the conserved region within the galectin carbohydrate binding domains. The asterisks (*) mark sequence identities between *G. cydonium* galectin-1 and -2 (GEODIA-1 and -2) and other galectins from *C. elegans* (CAEEL), eel (EEL), chicken (CHICK), mouse (MOUSE), rat (RAT) and human (HUMAN). The consensus sequence for galectins is given.

Monoclonal antibodies have been used as tools to identify the binding domains of the AF (Wagner-Hülsmann et al., 1996). A 140kDa polypeptide was found to participate in the AF-mediated reaggregation process. This polypeptide interacts with a galectin that links individual AF molecules to the AR at the plasma membrane, and consequently bridges two cells together (Müller et al., 1997) (Fig. 2A). Confocal laser scanning microscopical analysis demonstrated that both the galectin and the AF are present at the rim of the cells (Wagner-Hülsmann et al., 1997).

Within the last few years, major elements characteristic of a basal lamina have also been discovered in sponges. Besides cDNAs coding for two proteins with similar complexity as those found in higher Metazoa, collagen type IV, integrin, and one fibronectin module (FN3) were identified.

Integrin. One major class of extracellular matrix (ECM) receptors are the integrin receptors. Integrins are membrane-anchored heterodimer receptors composed of α- and β subunits. At least 16 different α- and 8 different β subunits have been identified in vertebrates which yield more than 20 heterodimeric integrin receptors.

We have isolated and characterised cDNA clones encoding the α subunit of an integrin from the marine sponge *G. cydonium* (Pancer et al., 1997a). The open reading frame encodes a 118,628Da polypeptide (Fig. 2B). Most α subunits of integrins, including the one from the sponge, contain 7-8 repeating domains (Fig. 2B). Like other α subunits of integrins the sponge molecule also contains putative divalent cationic-binding sites. A dendrogram was computed from the deduced aa sequences of integrin α subunits (Fig. 2C).

The integrin receptor binds primarily molecules of the following three families, present in the extracellular

consists of more than 10 type I-, approximately 2 type II-, and more than 15 type III (FN3) modules. Protein(s) have been isolated from *G. cydonium* that immunologically cross-react with human anti-fibronectin antiserum (Pahler et al., 1998). The main bands have sizes of 230 and 210kDa. In addition, a cDNA was cloned, encoding a putative 'multiadhesive protein' which comprises three interesting modules: 1) a fibronectin, 2) a scavenger receptor cysteine-rich (SRCR), and 3) a short consensus repeat (SCR) module (Fig. 3A).

The fibronectin module of the deduced sponge protein (Pahler et al., 1998) comprises the characteristic topology and aa found in fibronectin type-III (FN3) elements. Even though it remains to be proven in Porifera that this FN3 module functions as an adhesion molecule, the finding supports the immunochemical data on the presence of fibronectin-like molecules in sponges. FN3 modules have been primarily described in Metazoa; in addition they are found in a related sequence in extracellular glycohydrolases from soil bacteria (Bork & Doolittle, 1992). The unrooted phylogenetic tree (Fig. 3B) reveals that the FN3-related sequence of the bacterium branches off first from a common ancestor, while the deuterostomes (human and rat) and the protostomes (*Drosophila melanogaster* and *Schistocerca americana*) are grouped within one branch, and the acoelomate (*Caenorhabditis elegans*) and the sponge (*G. cydonium*) in another (Fig. 3B). From these data we conclude that the sponge FN3 module from *G. cydonium* is phylogenetically the oldest one within Metazoa.

Collagen. Collagens constitute a superfamily of extracellular matrix proteins. Until recently it was accepted that collagens are present only in Metazoa. However, a new class of collagens recently identified in fungi is assumed to have arisen by convergence (Celerin et al., 1996). In sponges, primitive fibrillar collagens have been seen in several species; *Chondrosia reniformis* (Garrone et al., 1975), and *G. cydonium* (Diehl-Seifert et al., 1985a). The finding that sponges contain basement membrane collagen type IV was spectacular, as this is known to be the scaffold of the basal lamina (Boute et al., 1996).

Galectin. As mentioned, the sponge AF interacts with the AR via galectin and the 140kDa polypeptide (Fig. 2A). The galectin, which occurs in isoforms, was studied in detail. The purified molecules reveal forms of Mr 13 to 18kDa (Bretting et al., 1981) which bind specifically to the sugars dGalNAc, dGalβ1→4dGlcNAc, dGalβ1→3dGlcNAc and dGalNAc. In the presence of Ca^{2+} or glycoconjugates the sponge galectins undergo conformational changes and 'polymerise' to large three-dimensional clumps (Diehl-Seifert et al., 1985b). The cDNAs of two isoforms of the galectins from *G. cydonium* were cloned (Pfeifer et al., 1993; Wagner-Hülsmann et al., 1996). The predicted proteins deduced from the complete sequences display high similarity with the corresponding molecules from vertebrates and *C. elegans* (Hirabayashi & Kasai, 1993; Müller et al., 1997). The deduced aa sequences of the two isoforms feature the characteristic carbohydrate-recognition domain **LHFNPR-G-V-N-W-E-R[H]**-PF (the aas given in bold are those directly involved in binding to the carbohydrate); this domain is conserved from sponge to human (Pfeifer et al., 1993) (Fig. 2D). Based on the extent of aa substitutions the two sponge galectins were calculated to have

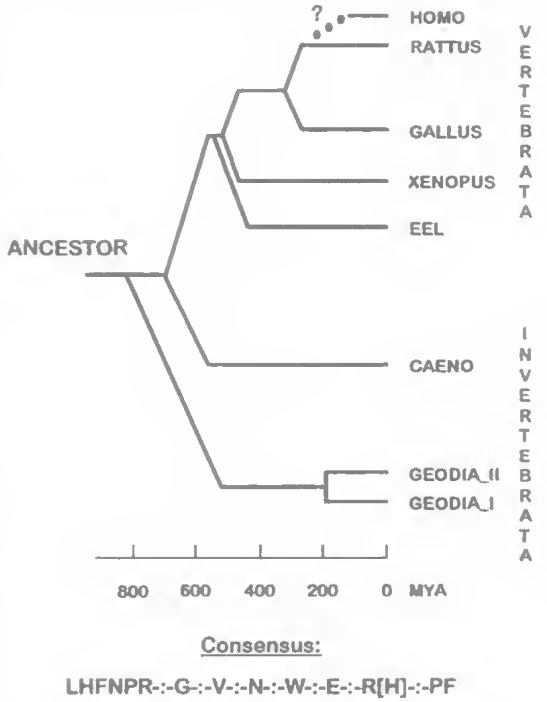

FIG. 4. Phylogenetic tree computed from the deduced aa sequences of galectins from: 1) vertebrates - human (HOMO), rat (RATTUS), chicken (GALLUS), frog (XENOPUS), conger eel (EEL); and 2) invertebrates - nematode (CAENO) and *G. cydonium* (GEODIA; isoform I and II). The consensus sequence for galectins are given. Time scale indicates the time of divergence, based on aa substitution analysis.

diverged from the galectin isolated from the nematode *C. elegans*, 800 MYA (Hirabayashi & Kasai, 1993; Pfeifer et al., 1993) (Fig. 4).

CELL-SURFACE RECEPTORS. Besides adhesion receptors, receptors involved in signal transduction, or elements of signal transduction pathways coupled to them have also been cloned from *G. cydonium*. The results have been recently reviewed (Müller & Müller, 1997; Müller, 1997a); so only a brief summary is given here.

One-transmembrane-segment receptor-receptor tyrosine kinase. Protein tyrosine kinases (PTKs) play important roles in the response of cells to different extracellular stimuli. PTKs are divided into two major groups, the receptor tyrosine kinases (RTKs), which are membrane spanning molecules with similar overall structural topologies, and the non-receptor TKs, also composed of structurally similar molecules. The first RTK from lower Metazoa was identified and cloned from *G. cydonium* (Müller & Schäcke, 1996). The putative aa sequence comprises 1) the extracellular part with a Pro/Ser/Thr-rich region, and two complete immunoglobulin (Ig)-like domains, 2) the transmembrane domain, 3) the juxtamembrane region, and 4) the catalytic tyrosine (TK)-domain. A similarity search with the *G. cydonium* TK-domain aa sequence showed that all RTKs fall in one branch of the tree while the non-receptor TKs are grouped in a second one; sponge RTK is placed in a separate branch, which splits-off first from the common tree of metazoan PTKs. An estimation of the time of divergence of the sponge RTK from RTKs of other metazoans was 650-665 MYA (Gamulin et al., 1997).

Seven-transmembrane-segment receptors. The first seven-transmembrane-segment receptor in sponges was isolated from *G. cydonium*. It is the metabotropic glutamate receptor (see description below).

Most seven-spanning receptors transmit extracellular signals through G-proteins. G-proteins, coupled to the putative receptors, have been identified in *G. cydonium*. G-proteins are heterotrimers composed of α-, β- and χ subunits (Seack et al., 1998).

Several secondary effector enzymes of the seven-transmembrane-segment receptor/G protein-linked receptor have been cloned from *G. cydonium*: the Ser/Thr kinases (STKs). These kinases are ubiquitously present in animal tissues; they express their activities in response to second messengers (e.g. Ca^{2+} or diacylglycerol). The STKs have been sequenced from *G. cydonium* (Kruse et al., 1996, 1997, 1998). A comparison of the complete structures of the sponge STKs, which are identical to those of nSTKs and cSTKs from higher Metazoa, with the structures of protozoan, plant and bacterial Ser/Thr kinases, indicates that the metazoan STKs are different from the non-metazoan enzymes. These data imply that metazoan STKs have a universal common ancestry with the non-metazoan STKs with respect to the kinase domain, but they differ from them in the overall structural composition.

NEURONAL-LIKE ELEMENTS IN PORIFERA. Until now no molecular evidence has been presented in demosponges for the existence of a nervous-like cell system. Recently our group identified two elements (crystallin and a metabotropic glutamate receptor) in sponges, which are characteristic for sensory systems in higher Metazoa.

Crystallins. Crystallins are categorised into two classes, the ubiquitous crystallins and the taxon-specific crystallins. No structural or functional characteristics are common to all crystallins. The α-, β- and χ-crystallins are classed as ubiquitous crystallins and are found in almost all vertebrate species. The second class, the taxon-specific crystallins, includes a series of 'enzyme crystallins', which display catalytic functions.

Until recently, no molecular sequence data was available for βχ-crystallins in invertebrates. The cDNA coding for the βχ-crystallin molecule was isolated from *G. cydonium* (Krasko et al., 1997). The sponge sequence comprises the four repeated motifs which compose the two domains of the βχ-crystallin. The peptide shows striking homologies to vertebrate βχ-crystallins. Each motif is composed of the four β-strands and one 'Greek key' signature (Fig. 5A). Like in other crystallins a signal peptide is missing in the sponge sequence, suggesting that it is an intracellular structural protein (Krasko et al., 1997). Thus, molecules from light-sensory organs, in this case crystallins, are also present in sponges.

Nerve cell receptors - presence of sensory cells: Metabotropic glutamate receptor. Sponges are (according to the literature) not provided with nerve cells. However, recently we showed that isolated cells from the marine sponge *G. cydonium* react to the excitatory neurotransmitter glutamate with an increase in the concentration of intracellular Ca^{2+}, $(Ca^{2+})_i$. This effect was also measured if the compounds L-quisqualic acid

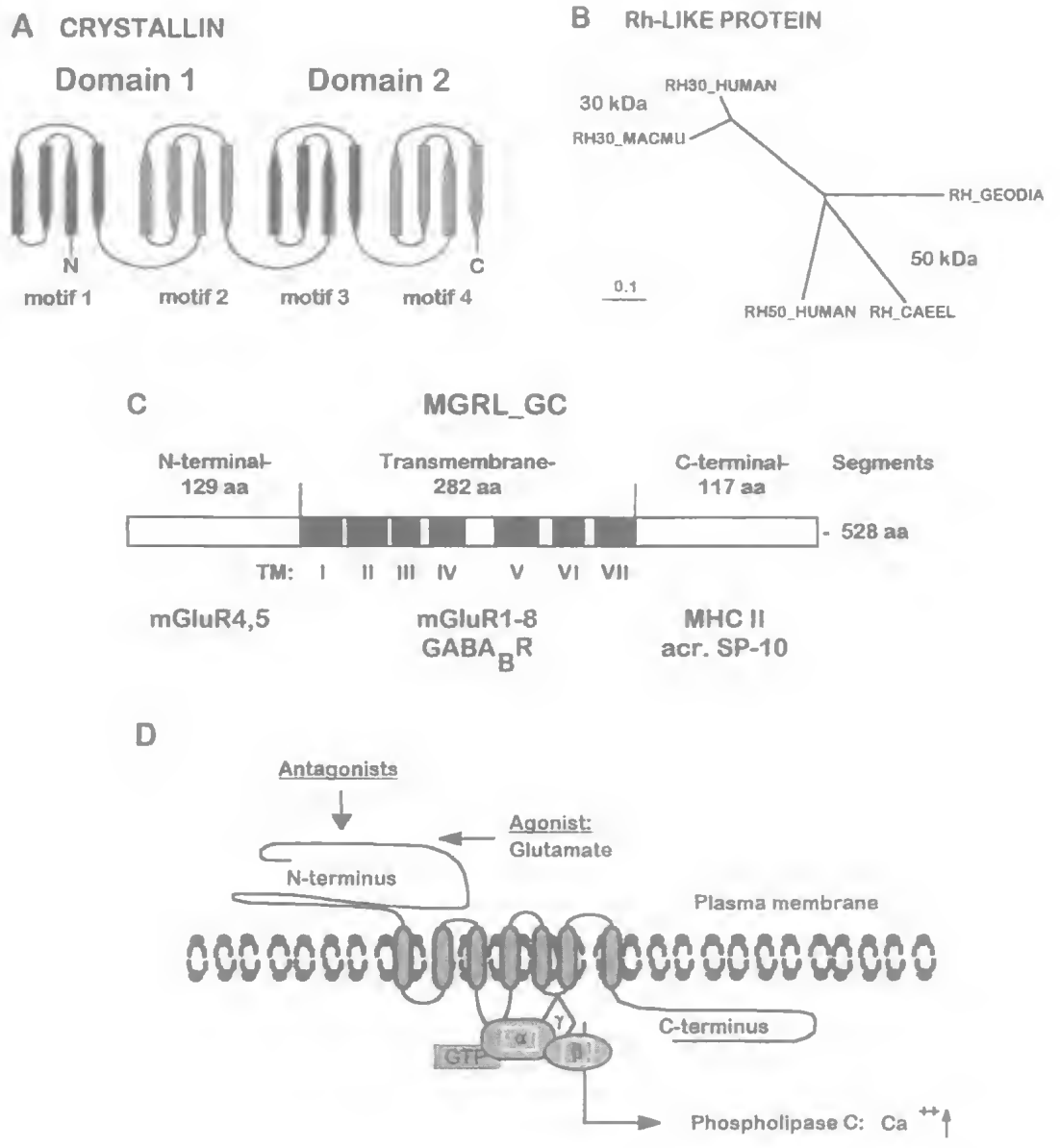

FIG. 5. A, Crystallin: Sponge βχ-crystallin from *G. cydonium*. Schematic representation of the βχ-crystallin folding pattern. Two Greek key motifs form one domain. Domains 1 and 2 form the monomeric βχ-crystallin. B, Rhesus-like antigen: Relationship of the sponge Rhesus(Rh)-like protein to animal Rh- and Rh-like antigens. Unrooted phylogenetic tree computed from the sponge Rh-like protein (RH_GEODIA) and Rh-related proteins: the Rh30 Ag from human (RH30_HUMAN) and rhesus monkey (RH30_MACMU), the RhD-like polypeptide from *C. elegans* (RH_CAEEL) and the human Rh50 Ag (RH50_HUMAN). Two clusters, comprising the ~30 000 Mr and the ~50 000 Mr Rh polypeptides, are grouped. C, Metabotropic glutamate receptors (mGluRs): Schematic presentation of the sponge receptor (*G. cydonium*), showing its three segments. The sequences with the highest similarity to the sponge segments are listed. D, mGluRs: Scheme of the sponge mGluR, inserted by seven transmembrane segments into the cell membrane; it is coupled to G-proteins.

(L-QA) or L-(+)-2-amino-4- phosphono- butyric acid (L-AP-4) were used. The effects of L-QA and L-AP-4, both agonists for metabotropic glutamate receptors (mGluRs), could be abolished by the antagonist of group 1 mGluRs, (RS)-α-methyl-4-carboxyphenyl- glycine. These data suggest that sponge cells contain a mGluR-like protein - hence it is justified to state that sponges are provided with sensory-like cells. The demonstration of a neuronal-like receptor in sponges also allows to challenge the question for the underlying molecules, involved in the coordination of the cells for contraction. It is interesting to note that some sponge species are known to migrate in a directed manner, e.g. the sponge *Tethya* spp (Fishelson, 1981) or *Ephydatia fluviatilis* (Bond & Harris, 1988).

Using a cDNA encoding the rat mGluR subtype 1, a complete nucleotide sequence of *G. cydonium* cDNA coding for a 528 aa long protein (59kDa) was identified which displays high overall similarity to mGluRs as well as to GABABRs. The deduced sponge polypeptide, termed a putative mGlu/GABA-like receptor, displayed the highest similarity to the two families of metabotropic receptors within the transmembrane segment. The N-terminal part of the sponge sequence shows similarity to the mGluR4 and -5. These findings suggest again that the evolutionarily earliest metazoan phylum, Porifera, possesses a complex intercellular communication and signaling system as known from the neuronal network of higher Metazoa (Perovic et al., 1999) (Fig. 5C and D).

HOMOLOGUES/MODULES OF THE IMMUNE SYSTEMS. Little is known about natural challenges to self integrity in sponges (reviewed in Pancer et al., 1996). In their extensive review Smith & Hildemann (1986) have grouped sponge alloimmune responses seen in experimental transplantations into two major rejection processes. Some species form barriers to separate themselves from non-self tissue, while others react by cytotoxic factors which destroy the transplant. However, until recently, no molecule has been identified which can be considered to be involved in self/non-self responses in sponges.

Three modules are now known in deduced aa sequences of cDNAs isolated from *G. cydonium*, which are present also in immune molecules of higher Metazoa; 1) immunoglobulin-like domains, 2) proteins featuring seavenger receptor cysteine-rich domains, and 3) molecules comprising short consensus repeats.

Immunoglobulin-like (Ig-like) domains. To determine if the two immunoglobulin-like (Ig-like) domains of the RTK from *G. cydonium* display sequence polymorphism (Pancer et al., 1996), allo- and autografting experiments were performed using two grafting methods: 1) parabiotic attachment, and 2) insertion technique. Thirty-six pairs of auto- and allografts were assayed. All of the autografts fused, while only two allografts fused and 34 pairs were incompatibile. At the molecular level the two Ig-like domains of RTK were analyzed from two pairs of fusing and one pair of rejecting sponges (Paneer et al., 1996). High nt and aa polymorphism was recorded.

Proteins featuring scavenger receptor cysteine-rich domains. Proteins featuring scavenger receptor cysteine-rich (SRCR) domains comprise a superfamily, which includes one invertebrate and several vertebrate proteins. The SRCR domain consists of a 110 aa-residue motif with conserved spacing of six to eight cysteines, which apparently participate in intradomain disulfide bonds. Proteins of this superfamily feature 1-11 SRCR domain repeats.

We identified the putative SRCR protein - belonging to group A of this family (Wijngaard et al., 1992) - from the marine sponge *G. cydonium* (Pancer et al., 1997b; Müller, 1997b). Three forms of SRCR molecules were characterised, which apparently represent alternative splicing of the same transcript. The long putative SRCR protein features twelve SRCR repeats, a C-terminal transmembrane domain and a cytoplasmic tail. The sequence of the short form is identical with the long form except that it lacks a coding region near the C-terminus, without the transmembrane domain. Homology searches revealed that the sponge putative SRCR protein shares with bovine T-cell antigen WC1 29.2% identity in 1054 aa overlap, 33.9% identity in 475 aa overlap with sea urchin speract, and 56% identity in 110 aa overlap with macrophage scavenger receptor type 1.

Recently, the SRCR module of the group B (Wijngaard et al., 1992) was also identified in the 'multiadhesive protein' (Pahler et al., 1998) (Fig. 3). The percentage of identity (homology) of this module of MAP_GEODIA is most similar to the mammalian sequences M130 with 63% (44%) and WC1 with 50% (40%) and lower for CD5 32% (25%) and CD6 36% (25%).

Phylogenetic analysis shows that the sponge MAP_GEODIA scavenger module branches off first from a common ancestor, whereas two other modules, the mammalian macrophage antigen M130 and the antigen WCI (which is expressed on gamma/delta T lymphocytes) as well as those of the CD6 and the CD5 antigen of lymphocytes, branch off later (Fig. 3C).

Molecules comprising short consensus repeats. The short consensus repeats (SCR) also termed 'Sushi domain', with 11-14 conserved aa residues and four conserved cystein residues, are classified according to the consensus aa pattern into four types. In the course of seeking further splice forms of the sponge putative SRCR, a protein sequence was identified which contains the 12 SRCR repeats mentioned above plus two others that are linked to six SCR, the SRCR-SCR molecule (Pancer et al., 1997b; Müller, 1997b). The SCR modules present in this putative polypetide belong to group II of the SCR family.

The presence of an SCR module of type III in a sponge molecule, 'multiadhesive protein', was surprising (Fig. 3A). This module belongs to the SCR repeats, which are dominant building blocks in the complement receptor of type 1, type 2, and factor H, but also in a few non-complement proteins (e.g. β2-glycoprotein I; reviewed by Reid & Day, 1989). The phylogenetic tree built from the selected SCRs of group III revealed that the sponge SCR module forms the basis for the two related SCRs from mammals, mouse complement receptor and human beta-2-glycoprotein I precursor, and the two invertebrate sequences from the locomotion-related protein of *Drosophila melanogaster* and the *Limulus* clotting factor (Fig. 3D). The SCR from the vaccinia virus displays closest relationship to the mammalian sequences, suggesting horizontal gene transfer from host to the virus (Bishop, 1981).

Rhesus-like protein. Vertebrate red blood cells display a variety of cell-surface molecules. Some, like the ABO system and the Rhesus (Rh) system of higher mammals, exhibit extensive polymorphism. Although the function of these antigens is poorly known, their role has been implicated in severe human disorders due to abnormal functioning or immunological destruction of the red blood cells (Nash & Shojania, 1987). A breakthrough in the analysis of the Rh system was marked by cloning of the Rh cDNA encoding the D antigen (Cherif-Zahar et al., 1990; Avent et al., 1990), and later also of the associated and closely related Rh50 protein (Ridgwell et al., 1992; Le van Kim et al., 1992). Surpringly, a Rhesus-like protein (cDNA) of 57,000Mr was isolated from *G. cydonium* (Seack et al., 1997). Both the hydropathy profile of the sponge Rh-like protein and its high similarity to the aa sequence clearly show that the sponge molecule shares a common ancestor with the human and rhesus monkey Rh30 antigen, and with the ~50,000Mr Rh-like polypeptides from humans and *Caenorhabditis elegans* (Fig. 5B).

CELL LINEAGES. In contrast to higher metazoan phyla, sponges are characterised by a pronounced plasticity in the determination of cell lineages. In a first approach to elucidate the molecular mechanisms controlling the switch from the cell lineage with a putative indefinite growth capacity to senescent, somatic cells, the activity of the telomerase as an indicator for immortality has been determined. The studies were performed on two demosponges, *Suberites domuncula* and *Geodia cydonium* (Koziol et al., 1998).

High activity for the telomerase in tissue of both sponges was found, reaching about 30% of that seen in telomerase-positive mammalian reference cells. In contrast, dissociated spherulous cells from *G. cydonium*, after an incubation period of 24hrs, contain no detectable telomerase activity. From earlier studies it is known that isolated sponge cells do not proliferate (Gramzow et al., 1989). From this it is assumed that the separation of the senescent sponge cell lineage from the immortal germ-/somatic cell lineage is triggered by the loss of contact to cell adhesion factors. Preliminary evidence exists which suggests that the final progress of the senescent, telomerase-negative cells to cell death is caused by apoptosis (Fig. 6).

ESTABLISHMENT OF A PRIMARY CELL CULTURE FROM A SPONGE. Despite the fact that cells from sponges contain high levels of telomerase activity, no successful approach to cultivate sponge cells has yet been described; in phyla which are evolutionary higher than sponges the somatic cells are telomerase-negative. One reason may be seen in the observation that after dissociation the cells lose their telomerase activity. In addition, no nutrients and metabolites have been identified that would stimulate sponge cells to divide.

In close collaboration with the group of R. Borojevic and M.R. Custodio (Departamento de Histologia e Embriologia, Instituto de Ciêcias Biomédicas, Universidade Federal, Rio de

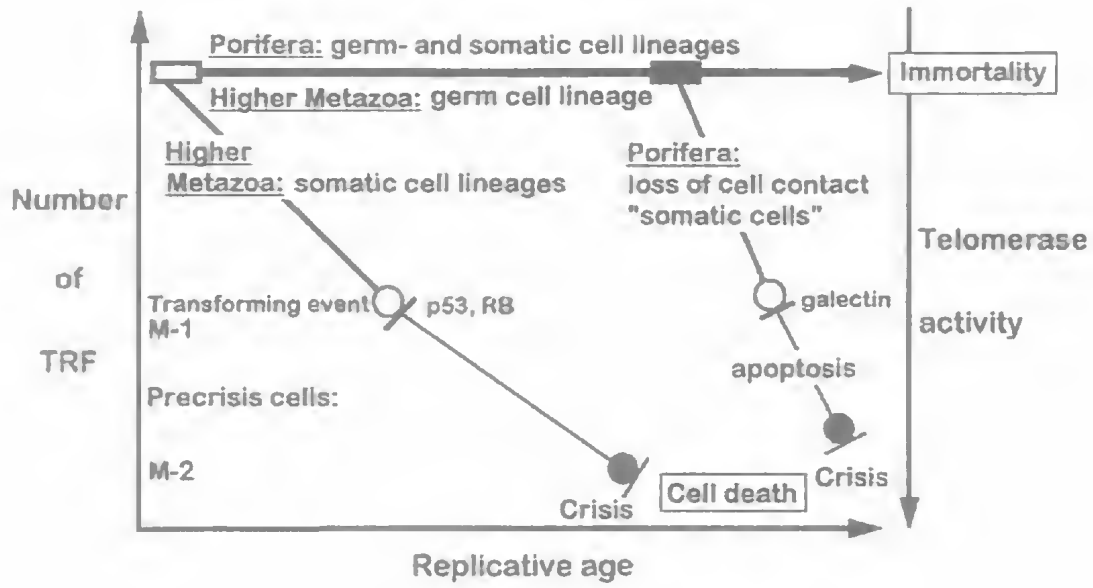

FIG. 6. Hypothetical consequence of telomere loss on senescence of metazoan cells. The reduction of telomeres is given in number of loss of terminal restriction fragments (TRFs). It has been shown experimentally that the cells of the germ line in higher Metazoa and the cells of both the germ- and the somatic lineage in Porifera contain high levels of telomerase thus allowing the maintenance of stable telomeres. In higher Metazoa an early loss of telomerase activity determines the fate of the somatic cells to senescence (open box) via the two phases. 1) 'Mortality Phase 1' (M-1) - cell cycle arrest - during which factors controlling life span via recognition of 'damaged' DNA, e.g. the RB protein or p53 protein, are activated. 2) After transformation (downregulation of RB and/or p53) the second process, 'Mortality Phase 2' (M-2), is initiated during which the telomeres reach in the precrisis cells a critical length from which a signal for cell death arises. In sponges it is proposed that the switch from immortal somatic cells to mortal 'somatic' cells occurs after a loss of adhesion factors for a given set of cells (closed box). The M-1 point is assumed to be reached after only a few rounds of cell replication. The growth arrest might be bypassed by addition of adhesion factor(s), e.g. galectin. The second phase towards cell senescence is supposed to be induced by central death signal(s), e.g. activation of the expression of the *MA-3* gene, which are under the control of either extrinsic- or an intrinsic factors. (Adapted from Harley, 1991; Harley et al., 1994).

Janeiro; Brazil), we succeeded in defining the culture conditions required for the formation of multicellular aggregates of *S. domuncula* from dissociated single cells; these are termed 'primmorphs', resembling organotypic cell cultures (Custodio et al., 1998; Müller et al., 1999). These aggregates, formed in seawater supplemented with antibiotics, have a tissue-like appearance, and have been cultured for more than five months. Cross sections through the primmorphs revealed an organised zonation into a distinct unicellular epithelial-like layer of pinacocytes and a central zone composed primarily of spherulous cells. After their association into primmorphs, the cells turn from the telomerase-negative state to the telomerase-positive state. Important is the finding that a major fraction of the cells in the primmorphs undergoes DNA synthesis and hence has the capacity to divide.

We propose that the primmorph system developed by us is a powerful novel model system to study basic mechanisms of cell proliferation and cell death; it can also be used in

aquaculture for the production of bioactive compounds and as bioindicator system.

SYSTEMATIC CONSIDERATIONS ON THE TWO SPONGE SUBPHYLA: HEXACTINELLIDA AND CELLULARIA. It has been proposed that Porifera should be divided into two subphyla, Cellularia (comprising Demospongiae and Calcarea), and Symplasma (containing only Hexaetinellida) (Reiswig & Mackie, 1983). This classification reflects the fact that species belonging to the Cellularia are composed of uninuclear cells, while those in the Hexactinellida have syncytial tissues (Mackie & Singla, 1983). This fundamental structural difference raises the question whether the ancestors of the Metazoa in general, and the Porifera in particular, were colonial flagellates or syncytial ciliates.

The cDNAs coding for proteins which have been used to establish the classification of subphyla within the Porifera, in particular, and the monophyly of Metazoa in general, came from two Demospongiae: *Geodia cydonium* (Jameson) (Demospongiae, Tetractinomorpha, Astrophorida, Geodiidae), *Suberites domuncula* (Olivi) (Demospongiae, Tetractinomorpha, Hadromerida, Suberitidae); one Calcarea: *Sycon raphanus* (Schmidt) (Calcarea, Calcaronea, Leucosoleniida, Sycettidae); and two Hexactinellida *Rhabdocalyptus dawsoni* (Lambe) (Hexactinellida; Hexasterophora; Lyssacinosida; Rossellidae), *Aphrocallistes vastus* (Schulze) (Hexactinellida, Hexasterophora, Hexactinosida, Aphrocallistidae).

THE PHYLOGENETIC POSITION OF THE TWO SUBPHYLA OF PORIFERA. Two alternative hypotheses have been proposed to explain relationships between the major sponge classes. One groups the Porifera into the adelphotaxa Hexactinellida and Demospongiae/Calcarea based on the gross difference in tissue structure and on differences in the structure of the flagellae, whose beating generates the feeding current through sponges (Mehl & Reiswig, 1991). The other hypothesis assumes that the Demospongiae are more closely related to Hexactinellida based on presumed larval similarities (Böger, 1988).

MOLECULAR APPROACH: PROTEIN KINASE C. In order to approach this question the cDNA encoding a protein kinase C, belonging to the C subfamily from the hexactinellid sponge *R. dawsoni*, has been isolated and characterised (Kruse et al., 1998). The two conserved regions, the regulatory part with the pseudosubstrate site, the two zinc fingers and the C2 domain, as well as the catalytic domain were used for phylogenetic analyses. Sequence alignment and construction of a phylogenetic tree from the catalytic domains revealed that the hexactinellid *R. dawsoni* branches off first among the metazoan sequences; the other two classes, Calcarea (using the sequence from *S. raphanus*) and Demospongiae (using sequences from *G. cydonium* and *S. domuncula*) branch off later. The statistically robust tree also shows that the two cPKC sequences from the higher invertebrates *D. melanogaster* and *Lytechinus pictus* are most closely related to the calcareous sponge (Kruse et al., 1998) (Fig. 7A).

This finding was also confirmed by comparing the regulatory part of the kinase gene.

MOLECULAR APPROACH: 70KDA HEAT SHOCK PROTEIN. Previous analyses of the 70kDa heat shock protein isolated from the same sponge species (Koziol et al., 1997) justify the conclusion that: 1) within Porifera, the subphylum Hexactinellida diverged first from a common ancestor to the Calcarea and the Demospongiae, which both appeared later; and 2) the higher invertebrates are more closely related to the calcareous sponges (Müller et al., 1998).

ADDITIONAL SUPPORT FOR TWO SUBPHYLA: INSULIN-LIKE RECEPTORS. Further support came from the analysis of the autapomorphic character restricted to all the Metazoa including Porifera, the transmembrane receptor tyrosine kinases (RTKs). Recently, we screened for the presence of molecules grouped into one specific subfamily within the superfamily of the RTKs (which includes the insulin receptors (InsR), the insulin-like growth factor 1 receptors and the InsR-related receptors), all found in vertebrates, as well as the InsR-homologue from *D. melanogaster*. The cDNAs, encoding the putative InsR-homologues, were isolated from the hexactinellid sponge *A. vastus*, the demosponge *S. domuncula* and the calcareous sponge *S. raphanus* (Skorokhod et al., submitted).

Phylogenetic analyses of the catalytic domains of the putative RTKs showed that sponge polypeptides have to be grouped to the putative InsR-homologues. Relationships revealed that all sponge sequences fall into one branch, while the related sequences from higher Metazoa, including the invertebrate sequences from insects and molluscs, or polypeptide(s) from one

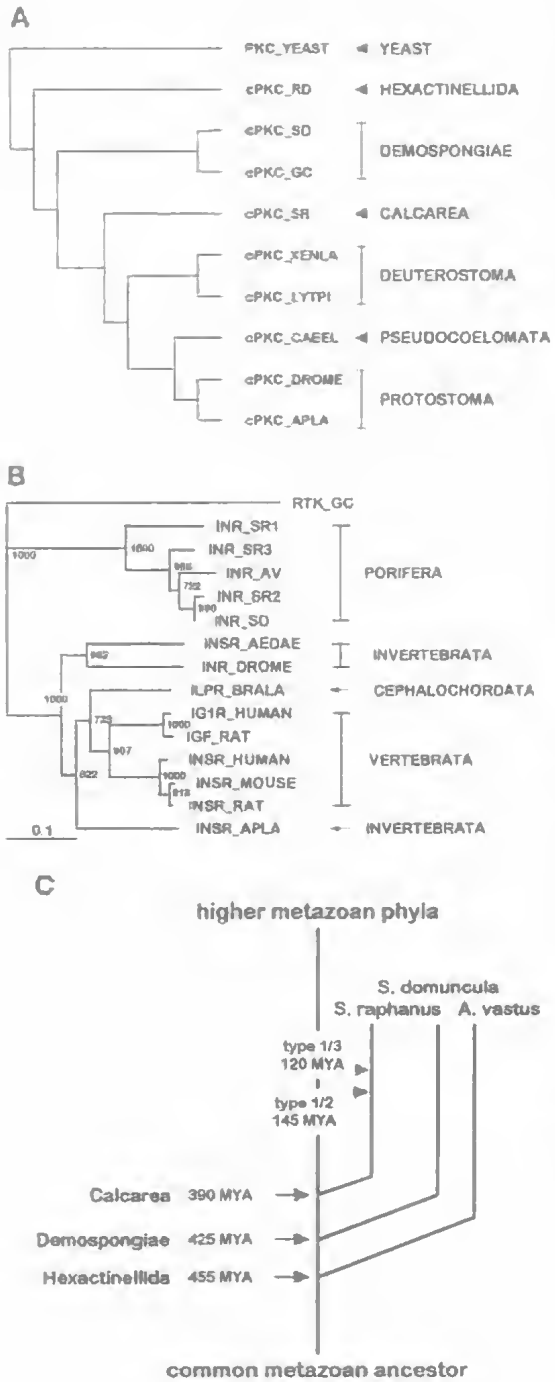

FIG. 7. Evolutionary position of Hexactinellida, Demospongiae and Calcarea. A, Phylogenetic tree based on the alignment of the catalytic domains of the deduced PKCs from: 1) Metazoa - cPKCs from the deuterostomes *Xenopus laevis* (frog - cPKC_XENLA) and *Lytechinus pictus* (sea urchin - PKC_LYTPI), from the protostomes cPKC *Drosophila melanogaster* (fruit fly - PKC_DROME) and *Aplysia californica* (mollusc, PKC_APLA); 2) Sponges of the classes Demospongiae, *Geodia cydonium* (CPKC_GC) and *Suberites domuncula* (CPKC_SD), Calcarea, *Sycon raphanus* (CPKC_SR), and Hexactinellida, *Rhabdocalyptus dawsoni* (CPKC_RD); 3) Yeast *Saccharomyces cerevisiae* (PKC_YEAST). B, Analysis of the insulin receptors (InsR), the insulin-like growth factor I receptors and the InsR-related receptors from vertebrates and invertebrates together with those from sponges. The deduced aa sequences of InsR homologues from the polypeptides of the three classes of Porifera: 1) Demospongiae: *S. domuncula* (INR_SD); 2) Calcarea *S. raphanus* type 1 (INR_SR1), *S. raphanus* type 2 (INR_SR2), *S. raphanus* type 3 (INR_SR3), and 3) Hexactinellida: *Aphrocallistes vastus* (INR_AV). All have been aligned with the related sequences for invertebrates: the insulin-like reeepior preeusor from the mosquito *Aedes aegypti* (INSR_AEDAE) and the InsR homologue from the fruit fly *Drosophila melanogaster* (INR_DROME) as well as the mollusc *Aplysia californica* InsR (INSR_APLA), one cephalochordate: the insulin-like peptide receptor precursor from amphioxus *Branchiostoma lanceolatum* (ILPR_BRALA) and from selected vertebrates: the human insulin-like growth faetor I receptor preeursor (IG1R_HUMAN), the human InsR precusor (INSR_HUMAN), the InsR preeursor from the house mouse *Mus musculus* (INSR_MOUSE), the IGF-I-R I receptor preeursor from the rat *Rattus norvegicus* (IGF_RAT) and the InsR precusor from *R. norvegicus* (INSR_RAT). The RTK domain from the sponge *G. cydonium* (aeeession number X77528) was used for comparison. The rooted phylogenetie tree of the catalytic domains of these sequences is shown. C, Proposed braneliing order of the three elasses of Porifera (Hexactinellida, Demospongiae and Calcarea), from a common metazoan aneestor. In addition, the separtion of the three types of the *S. raphanus* InsR-homologues, type 1 from type 3, and type 1 from type 2, are also indicated. The dates of the approximate divergence time are indieated (MYA).

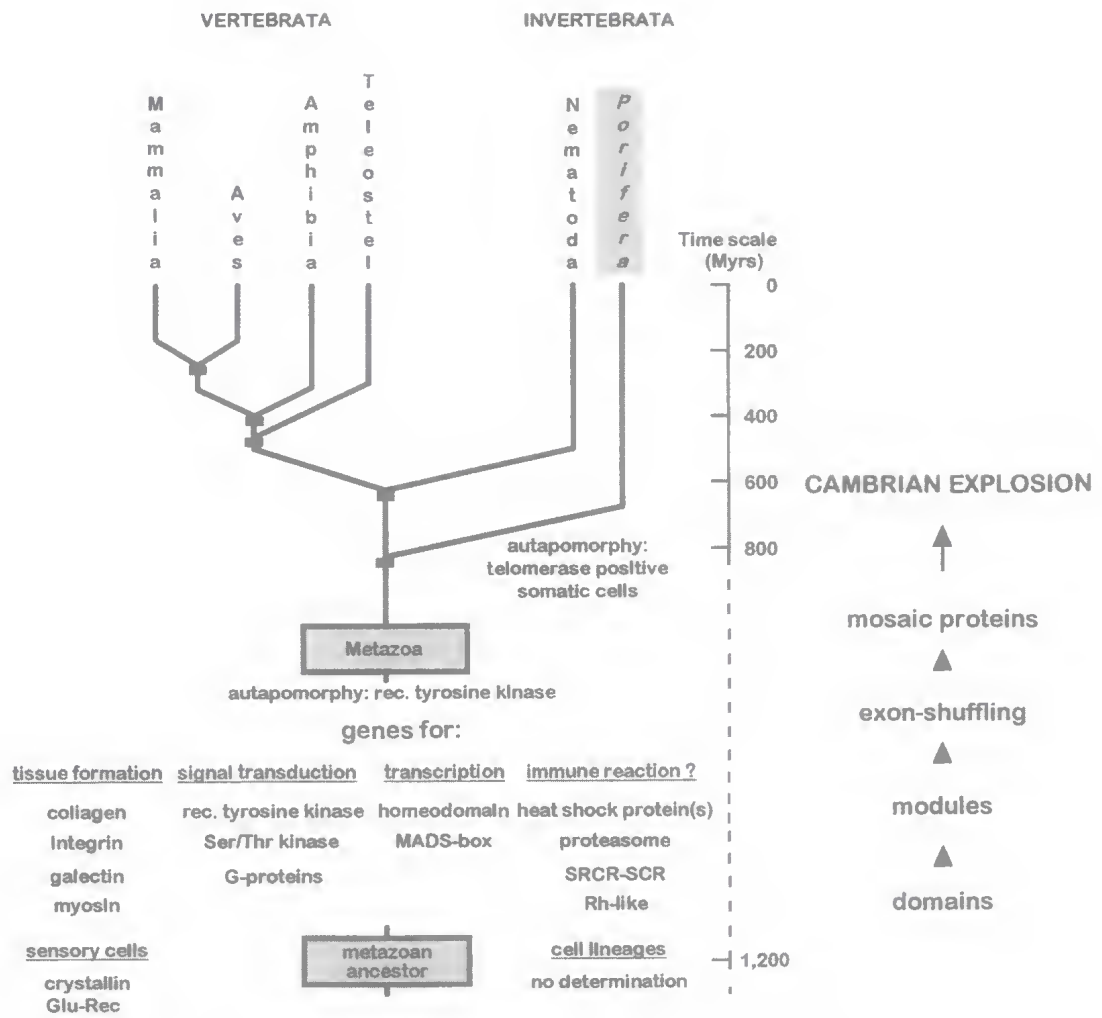

FIG. 8. Phylogenetic relationship of Porifera within the animal groups based on molecular biological data, obtained from sequences of 'metazoan' proteins required for tissue formation, signal transduction, transcription, immune reaction (potential) and sensory cells. The cell lineages in sponges are less determined than in higher Metazoa. It is proposed that the Cambrian explosion of metazoan radiation became possible after the creation of the evolutionary mechanism of modularisation of distinct protein domains, thus allowing the formation of mosaic proteins by exon-shuffling; this process happened approximately 1,000MYA. It is indicated that the presence of the telomerase activity is one autapomorphic character of Porifera.

cephalochordate and from selected vertebrates (human, mouse and rat) fall together into a second one.

Full length clones have been isolated from *S. raphanus*, that in addition to having the characteristic signatures for InsR-homologues, have one complete and one incomplete calcium binding epidermal growth factor receptor (EGF)-like domain in the extracellular regions.

Estimation of the rate of evolution of InsR-homologues revealed that the InsR-homologue of the hexactinellid sponge *A. vastus* is the phylogenetically oldest one (455MYA), while the molecules from the demosponge *S. domuncula* (425MYA) and the calcareous sponge *S. raphanus* (390MYA) are phylogenetically younger (Fig. 7B-C).

SPONGES AS LIVING FOSSILS. Based on the sequence data presented here it is reasonable to

state that Porifera should be placed into the kingdom Animalia together with the (Eu)Metazoa (Müller et al., 1994; Müller, 1995, 1997a). In addition, from the analysis of these first sponge genes, especially the one coding for RTK, it is now established that modular proteins, composed by exon-shuffling, are common to **all** metazoan phyla; a detailed description is given elsewhere (Müller & Müller, 1997). This mechanism of exon-shuffling is apparently absent in plants and protists (Patthy, 1995). If this view can be accepted then the burst of 'evolutionary creativity' (Patthy, 1995) during the period of Cambrian explosion, which resulted in the big bang of metazoan radiation (Lipps et al., 1992), was driven by the process of modularisation. During this process the existing domains were transformed into mobile modules, allowing the composition of mosaic proteins (Fig. 8).

As an example, the Ig-like domain is not an invention of Metazoa. Molecules featuring Ig-like domains appeared early in eukaryotic evolution (e.g. they are present in yeast a-agglutinin cell wall-associated protein; Chen et al., 1995). However, their use as modules, as building blocks, for the creation of mosaic proteins only became possible after a new step in evolution was acquired which allowed exon-shuffling. The mechanism of modularisation is more universal and more versatile - it can be applied to all preexisting domains - than the process of forming new domains. Therefore, it can be assumed that during the transition from Protozoa to Metazoa, a process which lasted approximately 1,000 million years, the formation of domains with distinct folds was at the center of evolution. After having reached a critical number of domains the mechanism of modularisation allowed a rapid formation of a series of mosaic proteins by exon-shuffling.

During the transition from unicellular Protista to multicellular Metazoa, the primary pattern of differentiation implies the presence of at least two different cell types, and as such the simplest multicellular organism could have consisted of one cell type specialised for feeding and the other for reproduction (Wolpert, 1990). This is in agreement with Roux-Weismann's original concept of primary separation of somatic and germinal cell lineages (Weismann, 1892), in which the immortal germen produces a mortal soma that will sustain the growth and reproduction of the organism but will necessarily perish. In view of the proposed monophyletic evolution of metazoans, and the position of sponges at the base of the evolution of multicellularity, we have addressed the question for those molecular mechanisms which underlie the evolution of the germ cell- and somatic cell-lineages, and the potential control of their immortality or their programmed senescence and death.

Sponges reproduce both asexually, by bud- and gemmule-formation, and sexually by production of gametes (reviewed in Simpson, 1984). But they lack special reproductive organs. The identification of putative stem cells for primordial germ cells in sponges has not been clearly provided, and the compelling morphological evidence for the origin of gametes from the somatic fully differentiated cells, such as choanocytes, argues against the clear separation of the germinal and somatic cell lineages. Preliminary experimental evidence has now been presented which reveals that sponge tissue is rich in telomerase activity, suggesting that the separation of cell lineages of somatic and germ stem cells has not been established, the determination of the fate of given sponge cells is still dynamic and might, under different physiological conditions, be reversible. It is proposed that the presence of telomerase activity is one autapomorphic character of Porifera (Fig. 8).

CONCLUSION

Our data show that sponges contain, as taken from deduced aa sequences, most structural elements known from higher Metazoa. It was intriguing to realise during the last three years that, while belonging to Metazoa, sponges 1) do have in some respect primitive, primordial metazoan characteristics, (e.g. simple elements of an immune system, with the 'multiadhesive protein' as an example), and 2) are already provided with complex and highly structurally evolved molecules not yet described from higher Metazoa such as the SRCR molecule. It is fortunate that sponges are not extinct. Assuming that Porifera were not the first metazoan phylum to evolve, they were witnesses to an evolutionary step that occurred during the maturation of the Metazoa near the Proterozoic-Phanerozoic boundary, close to 1 billion years ago. In this respect they can be considered to be living fossils.

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